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Sexing mammalian sperm for production of offspring: the state-of-the-art

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Abstract

Predetermination of sex in livestock offspring is in great demand and is of critical importance to providing for the most efficient production of the world's food supply. With the changes that have taken place in animal agriculture over the past generation the application of sex preselection to production systems becomes increasingly necessary. The current technology is based on the well-known difference in X- and Y-sperm in the amount of DNA present. The method has been validated on the basis of live births, laboratory reanalysis of sorted sperm for DNA content and embryo biopsy for sex determination. The technology incorporates modified flow cytometric sorting instrumentation to sort X- and Y-bearing sperm. Resulting populations of X or Y sperm can be used in conjunction with IVF in swine and in cattle for the production of sexed embryos to be transferred to eligible recipients for the duration of gestation. It can also be used for intratubal insemination and for deep-uterine and conventional insemination in cattle. This semipractical sexing method, though currently impractical for some production systems (where large numbers of sperm are required for fertilization) could be used to provide a more flexible progeny-producing option in many livestock operations. Improvements in the production rate of sexed sperm continue as new technology is developed. High-speed sorting is one of the newer technological advances and is being used in our laboratory to increase sorted sperm throughput. With our original technology we sorted 350,000 sperm/h. We now sort 6 million of each sex, under routine conditions. Sorting only the X population results in about 18 million sperm/h. Improvements in the technology will no doubt lead to much greater usage of sexed sperm, depending on the species involved. Insemination of lower sperm numbers in cattle has proven to be an effective means of utilizing the sexing technology. Solving the problems associated with inseminating low sperm numbers in the pig would be advantageous to the utilization of sexed sperm for some type of deep artificial insemination. Such a development would also enhance the economy of using lower sperm numbers with conventional artificial insemination (AI) and aid the swine industry world-

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wide. The use of sexed sperm for non-ordinary applications such as endangered species, laboratory animals, hobby or pet species is also of interest and will become a part of the move to be more reproductively efficient in the next millennium. Sexed sperm on demand over the next several years will provide livestock producers with many options in seeking to improve efficiency of production and improve quality of products to enhance consumer acceptability.

Keywords: Flow cytometer; Sperm sorter; Sexing; X–Y sperm; Livestock; In vitro fertilization; DNA

1. Introduction

Management schemes for livestock production can benefit from sex preselection because of the ability to plan matings for a specific sex. Making the sex determination prior to conception is the most cost-effective means of achieving the desired result. The only accurate and potentially cost effective approach for achieving sex preselection at the present time involves separating the X- from the Y-chromosome-bearing sperm followed by its use for artificial insemination (AI) or for in vitro fertilization (IVF) with subsequent embryo transfer (ET). Production of embryos followed by sexing in the laboratory is practiced to a limited extent, the inefficiency associated with discarding the embryos of unwanted sex limits its usefulness, unless a market has been established for both male and female embryos. In addition to more rapid genetic progress from the use of sexed sperm or embryos there are additional advantages of management and facility flexibility. One particular area of swine production that would benefit from sex preselection is the production of male and female hybrid lines of swine. Numerous other examples for specialized use of sex preselection also exist. As with the utilization of most assisted reproductive practices (IVF–ET), sexed sperm for production of livestock of preselected sex is dependent on efficiency, economics, effectiveness, and ease of use. Widespread use of sexed sperm will increase as each of these factors is considered and the technology is developed to such an extent as to become available on demand. At the present time economics and ease of use remain goals to be attained. Published results clearly prove the effectiveness and efficiency of the current sexing process in a broad range of applications (Johnson and Welch, 1999; Johnson et al., 1999; Seidel et al., 1999).

Since our first report utilizing DNA as a marker for separating viable intact X- from Y-chromosome-bearing sperm (Johnson et al., 1989) in which more than 50 rabbits were born of the predicted sex, more than 1500 offspring have been born in animals and humans using this method (Johnson and Seidel, 1999). The following references describe the applications of the flow cytometric sperm sorting as developed in our laboratory (Johnson, 1991; Cran et al., 1993, 1995, 1997; Rath et al., 1997, 1999; Seidel et al., 1997, 1999; Catt et al., 1996; Fugger et al., 1998). In the past couple of years several field trials have been conducted using more than 1000 heifers. These trials have again proven the efficacy of the technology under AI conditions in practical surroundings. The results from these trials clearly establish the method as one that can be used in practical cattle applications (Seidel et al., 1999). This paper will summarize the current state-of-the-art in sex preselection using flow cytometric sperm sorting for sorting X

from Y sperm based on DNA difference. At the present time there is no other method that has been repeatably proven to be effective in producing offspring of the predicted sex so the reader may want to consult other sources for reviews of various methods that have been attempted in seeking to sex sperm (Kiddy and Hafs, 1971; Amann and Seidel, 1982; Johnson, 1994, 1995).

2. Analysis for sperm DNA content the basis for X/Y sperm separation

It was shown early in the 20th century (Guyer, 1910) that sex chromosomes were unique in the overall chromosome complement. In the 1950s it was determined that DNA was a critical component of the sex chromosomes. Morruzi (1979) was the first to suggest that DNA might be used as a differentiating factor of sex chromosomes and that it might provide the basis for separating X- and Y-chromosome-bearing sperm. The fact that the X chromosome of most mammals carries more DNA than the Y chromosome was clearly established from those studies (Morruzi, 1979). The Y chromosome is smaller and carries less DNA than the larger X chromosome, while the autosomes carried by X- or Y-bearing sperm are identical in DNA content. Taking advantage of this real difference for purposes of X- and Y-bearing sperm separation has only been possible since the advent of flow cytometry. The first attempts to differentiate X- and Y-sperm using DNA and flow cytometric analysis did not succeed (Gledhill et al., 1976). However, with the combination of improved staining methods and a realization that aspherical cells such as sperm must be orientated to the excitation source the relative DNA difference could be measured (Pinkel et al., 1982). These factors formed the basis for routine sperm DNA analysis using modified commercial instrumentation (Johnson and Pinkel, 1986) and the demonstration that sperm sex ratio could be validated by analytical methods (Johnson, 1988; Johnson et al., 1987a; Welch and Johnson, 1999). Sorting sperm into separate and nearly pure X and Y populations based on the DNA difference was accomplished with the modified flow cytometry/cell sorter instrumentation (Johnson and Clarke, 1988; Johnson et al., 1987b). However, these reports had a key factor in common with the analysis of sperm DNA mentioned in the previous section, the sperm were dead because they had been sonicated to remove the tails (versus intact motile sperm). As a prelude to the ability to sort living sperm, the sperm nuclei were sorted and used for injection into the cytoplasm of the hamster egg (Johnson and Clarke, 1988). This study proved the viability of the DNA of the sorted sperm head for fertilization even though the sperm under standard conditions could not fertilize on its own.

3. Sorting sperm: modifications required in order to sort X- and Y-chromosome-bearing sperm

Improved cellular staining and the utilization of vital fluorochromes to label the DNA of living sperm led to the sorting of X- and Y-chromosome-bearing sperm for the production of offspring of predetermined sex (Johnson et al., 1987a, 1989). Unlike

analysis of sperm for DNA difference indicated above, the sorting of sperm into separate populations of X- and Y-bearing sperm based on DNA requires a flow cytometer/cell sorter with a modified configuration. The cell sorter which incorporates the analytical flow cytometer must be modified in commercial cell sorters in order to properly measure DNA difference in X- and Y-bearing sperm (Johnson and Pinkel, 1986). Although fluorochromes designed to bind to DNA generally give off a bright signal, the 5-W water-cooled argon laser remains the laser of choice for most DNA fluorochromes. Cell sorters fall into two categories at the present time. The older class of sorters could be termed 'standard-speed' systems, where the samples are sorted under 0.84 kg/cm^2 of pressure and one can sort about 350,000 sperm/h (Johnson et al., 1989). The newer generation sorters are the 'high-speed' cell sorters that have come on the commercial market over the past 4 years and operate at sample pressures that range from 0.84 kg/cm^2 to 4.22 kg/cm^2. This sorting system modified for sperm can produce 6 million X sperm and 6 million Y sperm per hour at the present time. Even greater output can be achieved by sorting only X sperm (85% to 90% purity) at about 11 million/h (Johnson and Welch, 1999). The overall limits of this high speed system for sorting sperm has not been tested to the fullest extent yet, so it is likely that these values will move higher in the near future.

Fig. 1 illustrates a flow cytometer/cell sorter system configuration that has been modified specifically for sperm. Cell sorters normally are configured with a fluorescence detector perpendicular to the laser beam (90°). The modification consists of a forward fluorescence detector in place of the light scatter detector that is standard to orthogonal configured flow systems. This is necessary in order to collect the fluorescent light from both the edge of the sperm (90°) as well as from the flat side (0°) of the sperm. Through electronic gating based on collecting edge fluorescence the variability associated with differential fluorescence is removed and the two populations are resolved. Resolving X- and Y-bearing sperm based on DNA content using flow cytometric sorting is difficult when compared to other cell types. The inordinate compactness of chromatin in the morphologically flat, paddle or ovoid shaped sperm head that is characteristic for domestic animals, causes a high index of refraction. The difference in refractive index between the sperm head and the surrounding medium, coupled with the flat shape of the sperm head, results in preferential emission of light in the plane of the cell (from the edge of the sperm head). Because of these properties, the orientation of the sperm head with respect to the excitation laser beam and the optical detectors is critical for resolution. It is also essential for reanalyzing sorted sperm for DNA to determine the proportions of X or Y sperm in a given sorted sample (Johnson et al., 1987b; Welch and Johnson, 1999). The second part of the modification was initially designed around a beveled needle which produced a flat sample stream which allowed the sperm head to take up the plane of the stream and be oriented to the laser beam (Johnson and Pinkel, 1986). This was effective but inefficient only orienting 25% of the sperm. However, all of our early development work was done on this system (Johnson, 1994, 1995).

An improved orienting system (nozzle) was first fitted to the standard speed sorter. This improvement in orientation on the modified cell sorter through replacement of the beveled needle system with an orienting nozzle was reported by Rens et al. (1998). This has increased the percentage of sperm oriented from 25% to 70%. This enhancement has

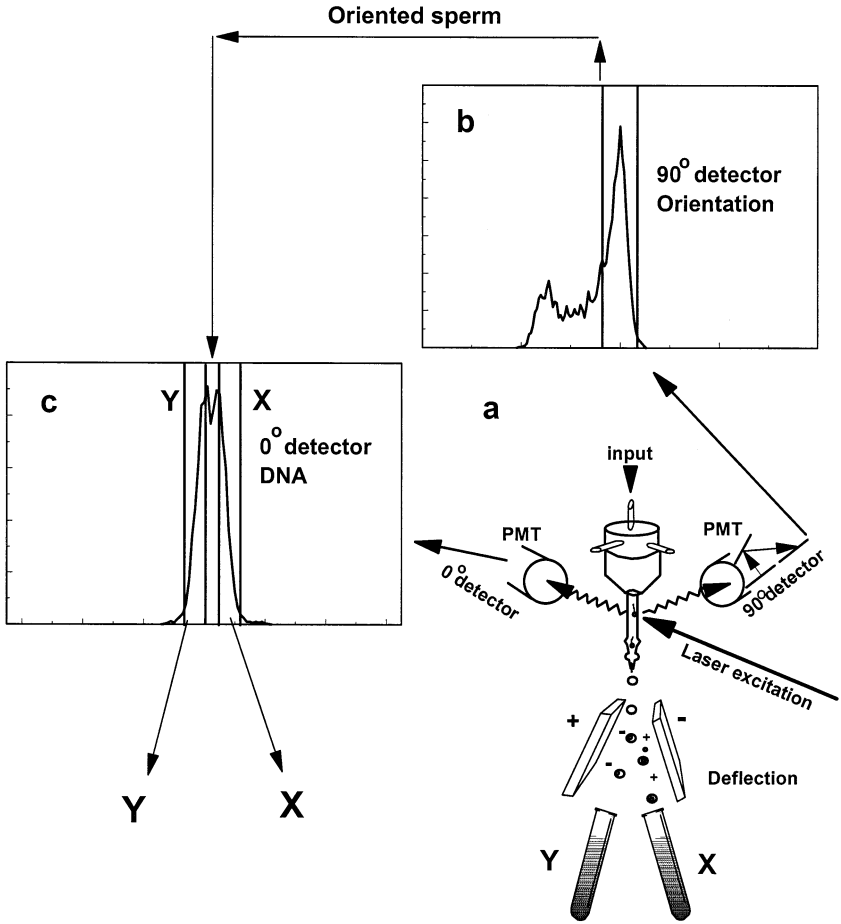

Fig. 1. A schematic diagram of the flow cytometer/cell sorter modified for sorting sperm (Johnson and Pinkel, 1986). Represents the current technology in high-speed sorting for resolution of X- from Y-chromosome-bearing sperm based on their difference in DNA content according to Johnson et al. (1989, 1999). Sperm enter through a needle labeled > sample input = and are passed into the flow cell (a). This needle formerly was beveled at the exit point to create a flat stream so as to control the orientation of the sperm to the laser beam. The current high-speed sorting systems utilize a cylindrical needle and an orienting nozzle (Ren et al., 1998) that more efficiently orients the sperm to the laser beam. Orientation occurs when the opposing flatter surfaces of the sperm head simultaneously face the laser and 0° forward detector, with the edge directed to the 90°C. (b) Depicts the fluorescence distribution from the 90° detector. Note the population within the vertical gated area, those are the portion of the sperm that are properly oriented (70%) to the laser beam. Histogram (c) illustrates the separation of the X and Y populations from the oriented population of histogram in (b). The partially resolved bimodal peaks have a sort window represented by vertical lines on the peaks. The area between the two sort windows is sperm that are mixed (X and Y) in varying percentages and so they are discarded through the waste. Sperm containing the smaller Y chromosome and thus less total DNA comprise the dimmer (left) population and similarly the larger X-chromosome-bearing sperm comprise the brighter population (increasing fluorescence). (a) Represents the overall sorting process, depicting the electrostatic field through which the charged droplets containing X or Y sperm must fall and be deflected to the waiting tubes.

improved overall efficiency of the sorting system by two- to threefold. The modified flow cytometer and cell sorter is essential for attaining separate populations of X- and Y-chromosome-bearing viable (Johnson et al., 1989) sperm on a repeatable basis. Differentiation of the amount of DNA present in the X- and Y-chromosome-bearing sperm for sorting can be done on virtually all commercial cell sorters that have been manufactured in the past 25 years if they have the basic modifications for sperm (Johnson and Pinkel, 1986).

4. High-speed viable sperm sorting

High-speed technology has the capability to increase sorting speed by 10 times over previous standard speed sorters. The MoFlo® high-speed cell sorter produced by Cytomation, (Ft. Collins, CO) was introduced in 1996. This system has the capability of sorting cells at much faster rates and at much higher pressures and is appropriately classed as a 'high-speed cell sorter'. Instead of the standard rate of 5000–10,000 events/s it has the capability of 50,000 events/s. The MoFlo®, as is the case with standard speed cell sorters, still must be modified to make it a sperm sorter (Johnson and Pinkel, 1986) in order to resolve the small differences in DNA content in X- and Y-bearing sperm. This high-speed system is what is currently in use in my laboratory for sorting viable sperm (Johnson et al., 1999); the principle of sorter operation remains the same. However, it lends itself to greater ease of use and the obvious faster sorting rate. It is equipped with the standard modifications in addition to the orienting nozzle. This high-speed sperm sorting system with orienting nozzle is capable of processing more than 40,000 events/s.

5. Sorting of viable sperm for production of sexed offspring

5.1. Preparation of the intact sperm sample for flow sorting

The most critical aspect of preparing sperm for use in sperm sexing is that the less insult imposed on the sperm, the greater the likelihood that the ultimate population of sorted sperm will keep and maintain their fertilizing capacity (Johnson and Welch, 1999; Johnson et al., 1989). Examples of insults imposed are: adding stain to the sperm, rewarming the sperm by incubation of the stained sperm, subjecting the sperm to pressure changes in the sorting system and centrifugation of sorted sperm. Using high speed sorting, one prepares an aliquot of 1 ml containing about 150 million sperm or 10 times the amount with the standard-speed sorter. Proportionally the same amount of Hoechst 33342 (bisbenzimide; Calbiochem-Behring, La Jolla, CA) is added at the rate of 8 μl/ml (5 mg/ml stock). The suspension is then incubated at 35°C for approximately 1 h. The incubation assists the penetration of the stain through the membrane. Uniform stain penetration is essential to minimize stain variation and helps to reduce the CV of the sperm separation. The result of proper processing is improved efficiency of sorting.

5.2. Collection of separate populations of X- and Y-bearing sperm

The need for maintaining viability of the sperm is critical right up through the collection of the sorted sperm. An environment that will minimize the dilution that accompanies cell sorting is essential. The concentration of the stained sperm to be sorted are lower (150 million/ml) compared to the original ejaculates (~300 million/ml for swine; ~one billion/ml for cattle). Once the fluorescently stained sperm pass into the flow cell, the stream is surrounded by a sheath fluid of phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) which contributes to an increasing dilution effect. The BSA helps to minimize agglutination of the sperm. Minimizing dilution is accomplished by sorting sperm into a tube containing an egg yolk extender at the outset. The TEST-yolk (20%) has been the very successful for this purpose (Johnson, 1991; Johnson et al., 1989). The procedure currently utilizes 50 μl of TEST-yolk per 0.5-ml tube size in standard sorters. For high-speed sorters the amounts are increased proportionally, that is, a 15-ml tube should contain 500 μl of TEST-yolk, which provides a concentrated medium for sperm to migrate to. The fluid volume in the tube increases as sorting progresses. The sperm are falling or being projected into the tube and the motile sperm continue their downward movement into the TEST-yolk extender at the bottom of the tube and remain there in a concentrated environment. Once sorting is terminated, sperm are centrifuged ($350–750 \times g$ depending on tube size) for 10 min to concentrate for insemination in vivo or in vitro depending on the species. All sorting takes place at room temperature.

The use of the sort collection process described above is most effective for sperm to be used for insemination. When using sperm for IVF to produce sexed embryos, the yolk percentage in the TEST-yolk can be reduced to less than 5% so as not to interfere with fertilization during IVF. A concentration of 2% yolk is sufficient to provide a concentrated environment for sorted sperm (Rath et al., 1997) to be used for IVF.

The use of seminal plasma as a portion of the collection fluid is also a possibility for minimizing the dilution effect. In several experiments, Maxwell et al. (1996) and Maxwell and Johnson (1997) showed that 10% heterologous seminal plasma as part of the TEST-yolk (2%) reduced the percentage of sperm showing the acrosome reaction after sorting. However, additional results using the seminal plasma protocol in combination with IVF in the pig showed that fertilization by sorted sperm was inhibited when the sperm were collected into a TEST-yolk (2%) medium containing 10% seminal plasma (Maxwell and Johnson, 1997). Premature capacitation is clearly a characteristic of sex sorted sperm, just as it is with the boar sperm processed for freezing (Pursel and Johnson, 1975). Though it tends to be disadvantageous for sperm being used for storage, it is advantageous for sperm being used soon after sorting for IVF as the sperm are already capacitated.

Although 100% purity of livestock sperm X or Y populations is nearly impossible to achieve with flow sorting under normal sorting conditions, one can easily approach 95% purity (Johnson, 1992; Rath et al., 1999). In some species where the DNA difference is greater, such as the *Chinchilla langier* (Fig. 2; 7.5%; Johnson et al., 1987b) 100% purity in the sort may be possible. Highest purities are attained in flow sorting when the DNA difference is greater than 3.5%, sperm are efficiently oriented to the laser beam and

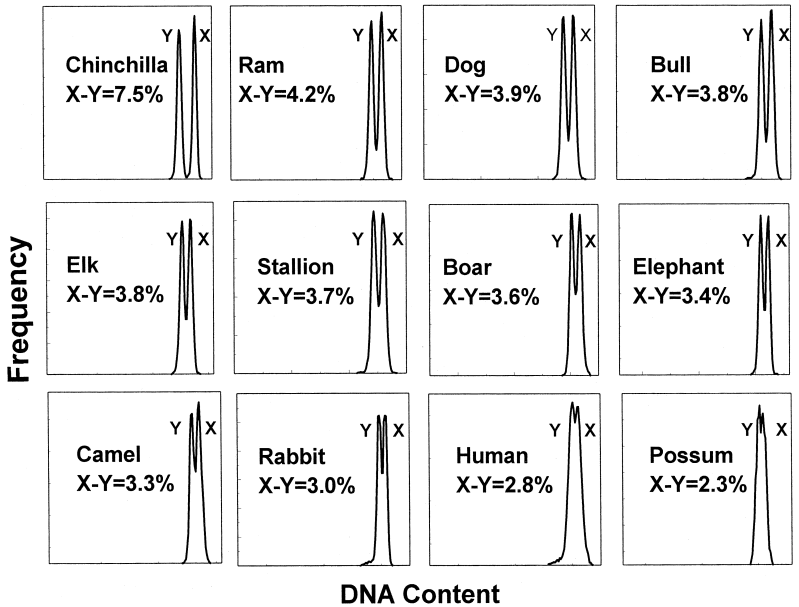

Fig. 2. Illustration of typical histograms from various species showing the respective differences in DNA between the X- and Y-chromosome-bearing sperm. Generally the wider the difference, (e.g. Chinchilla at 7.5%) the easier it is to sort at higher purity. Compare the human with a 2.8% difference in DNA, which is more difficult to sort at higher purity. (Adapted from Johnson and Welch, 1999).

there is uniform staining. Sorting human sperm presents more of a challenge since the DNA difference of X- and Y- sperm is about 2.8% (Fig. 2). Routinely the percentages of purity range from 75% to 90% for sorted human Y and X sperm respectively (Johnson et al., 1993; Fugger et al., 1998). Histograms for 12 different mammals are shown in Fig. 2. The depth of the split between the X and Y peaks on the histograms gives an indication of how easy it is to sort highly pure ($>90\%$) populations. However, other factors, such as dead sperm, abnormal morphology and the shape of the head may impact the sorting process. This can be illustrated with the possum and human sperm. At 2.3% the possum can be separated, however if the more angular shaped human sperm carried only a 2.3% difference, it could not be sorted with any consistency.

5.3. Validation of sorted X- and Y- sperm populations in the laboratory by sort reanalysis

At some point during a sort or immediately after the completion of a sort to be used for insemination or IVF, a presiliconized and/or BSA presoaked tube is placed in position to collect the sorted sperm. No TEST-yolk is added to the tube for the sort reanalysis. Approximately 100,000 sperm are sorted into each tube. After the aliquot has been sorted, the sperm are sonicated to remove the tails and Hoechst 33342 added to maintain staining uniformity and then flow cytometrically reanalyzed but not resorted. A detailed procedure for sort reanalysis that we developed at the outset of the project in 1982 has been recently published (Welch and Johnson, 1999). The proportion of X- and

Y-bearing sperm are determined based on the DNA difference and histograms are analyzed (Johnson et al., 1987a) by computer fitting to double Gaussian peaks (Fig. 1c). The other method of determining the sperm sex ratio is to use fluorescence in situ hybridization (FISH) procedures where the fluorescent signal can be counted to determine the proportion of Y-chromosome-bearing sperm carrying the Y microsatellite DNA probe. In a comparison on boar semen, we found no significant difference between the accuracy of the testing sort purity by FISH or DNA reanalysis (Kawarasaki et al., 1998). The polymerase chain reaction (PCR) has been found to give useful information about the proportions of X- and Y- sperm in a sorted sample. It can be used effectively based on sorting single sperm into 48-well plates and carrying out the PCR reaction (Welch et al., 1997). Sort reanalysis for DNA has an advantage over FISH and PCR since PCR and FISH may take from 3 to 4 h. Many investigators carry the procedure into the next day. Certain cell types are amenable to FISH at even faster periods; however, sperm require at least 3–4 h for FISH (Fugger et al., 1998) while sort reanalysis can be done within a 30-min time period (Welch and Johnson, 1999).

6. Fertility results using flow sorted X- and Y-chromosome-bearing sperm

6.1. Sperm sorted by standard-speed modified cell sorter

The first evidence of the efficacy of sorting sperm based on DNA content of the X- and Y- sperm was published in 1989 (Johnson et al., 1989). Litters of rabbits were produced that showed a significant skewing of the sex ration (81–94%). Intratubal insemination was tested next and again, litters of pigs were produced after surgical insemination of females with skewed sex ratios (Johnson, 1991). Initial work in our laboratory in the pig utilizing IVF resulted in two litters of pigs being born with all pigs born being of the predicted sex (Rath et al., 1997). Cleavage rates after IVF were 56% ($n = 367$) and resultant two- to four-cell embryos were transferred to asynchronous ($n = 4$) gilts. All embryos transferred were produced from IVF using X sorted sperm and all offspring were female. Improvements in IVF systems for the pig are required to make use of sexed sperm applicable. One factor that needs to be controlled carefully in using sexed sperm for IVF is that the sperm be used for IVF insemination soon after sorting. This need is based on the premature capacitation that is induced by the sorting process. It is important to sort the sperm over a 2- to 5-h period and use them for coincubation within 1–2 h if possible. It is possible though to use them after a longer period (Abeydeera et al., 1998); however, the percentage of viability may be significantly reduced. The IVF procedures are more advanced for cattle (Cran et al., 1993). Sperm sorted for IVF in cattle and subsequent transfer was effectively done with good results. In a field trial conducted in England and Scotland, bull sperm were sorted, used for IVF and the sexed embryos frozen for transfer on various farms. The offspring born from this study were 90% male which was what was predicted based on the sort reanalysis of the sperm used for IVF (Cran et al., 1995).

The availability of sexed sperm to the swine industry as well as for the cattle industry is needed in order to realize the great potential from livestock produced on the basis of sex. At the present time IVF and subsequent embryo transfer provide one means for

producing sexed litters of pigs. Another means available is using surgical insemination or laparoscopy in the pig for delivering sexed sperm to the site of fertilization (Johnson, 1991). Conventional AI techniques in the pig require about 3 billion sperm per dose of semen making it virtually impossible to use it for sexed sperm. Considerable effort is currently being put forth to develop methods for low dose insemination in the pig. Recent results using a type of endoscope to navigate the uterine horn and place small numbers of sperm near the utero-tubal junction show considerable promise for the future use of sexed sperm (Vazquez et al., 1999). Additionally several experiments investigating the appropriate numbers of sperm by which fertilization can be obtained in the pig have shown encouragement that as few as 10 million sperm placed in the upper part of the uterus resulted in litters born (Krueger et al., 1999). Recently we have conducted preliminary studies in which we used a Melrose catheter into the cervix of sows and then using a smaller internal catheter that extended into the distal uterus where the semen was deposited. Pregnancies were obtained with 100 to 400 million sperm per estrus in 8 sows (Johnson et al., 2000b). This is an area that could benefit greatly from more research. Indeed, the whole swine AI industry would benefit from greater research emphasis, since the increasing use of AI will bring about increasing pressure for greater economy of sperm per insemination dose. Perfection of a low dose method where 5 million or less sperm could be used per AI would likely signal the beginning of widespread use of sexed sperm for the pig.

Low number insemination in the cow has already proven successful both with sexed and unsexed sperm (Seidel et al., 1997). Pregnancies can be obtained using as little as 2×10^5 per insemination. Average percentage of the correct sex born based was 83% of the sex desired. This initial study using deep AI has been instrumental in the commercialization of sexed semen through AI of cattle (Seidel, 1999).

6.2. Offspring born after using sperm sorted for sex on a high-speed sorter modified for sperm

High-speed cell sorting is a significant technological improvement over standard speed cell sorters. As indicated above, we have modified the flow cell of the MoFlo® cell sorter to receive an orienting nozzle which provides an increase in oriented sperm from the previous beveled needle orientation of 25–30% to $\sim$70%. Two studies were conducted using sexed boar sperm produced by high-speed sorting/orienting nozzle for IVF in swine. However, in two studies conducted with the high-speed sorter, we used in vitro matured swine oocytes for fertilization under IVF conditions. An experiment was conducted at Beltsville and produced offspring in nine litters. Six litters produced from sows in which sexed embryos had been transferred at the four-cell stage gave 34 total piglets, of which one was male. The percentage was 97% female sperm Control litters (three) gave 52% male and 48% female offspring (Rath et al., 1999). Litter size averaged 7.8 and 5.8, respectively. Another experiment was conducted in collaboration with the University of Missouri. Sperm were collected and sorted into X and Y populations at Beltsville and shipped by air to Columbia, MO where they were used at the University of Missouri in their IVF system to produce sexed embryos. There were eight litters born from that study. From the X sorted sperm, 23 females and one male were born (97%) and from the Y sorted sperm nine pigs were born, all were male

(100%; Abeydeera et al., 1998). These two studies clearly demonstrate the efficacy of high-speed sperm sorting in conjunction with a special orienting nozzle to orient a greater percentage of sperm from each sample.

Extensive field trials using high-speed technology essentially as described here have also been conducted by Seidel et al. (1999) in collaboration with XY, (Ft. Collins, CO) over the past couple of years. One thousand heifers have been inseminated with sexed sperm and 370 were inseminated with control sperm. All inseminations were done after synchronizing estrus in the heifers. Accuracy of producing a male or female as determined by the sorted sperm that was inseminated approached 90%. Pregnancy rates were within 90% of the unsexed controls. Uterine body insemination was as successful as insemination bilaterally into the uterine horns. The results of these studies with cattle illustrate the effectiveness of using uterine insemination and conventional AI making the

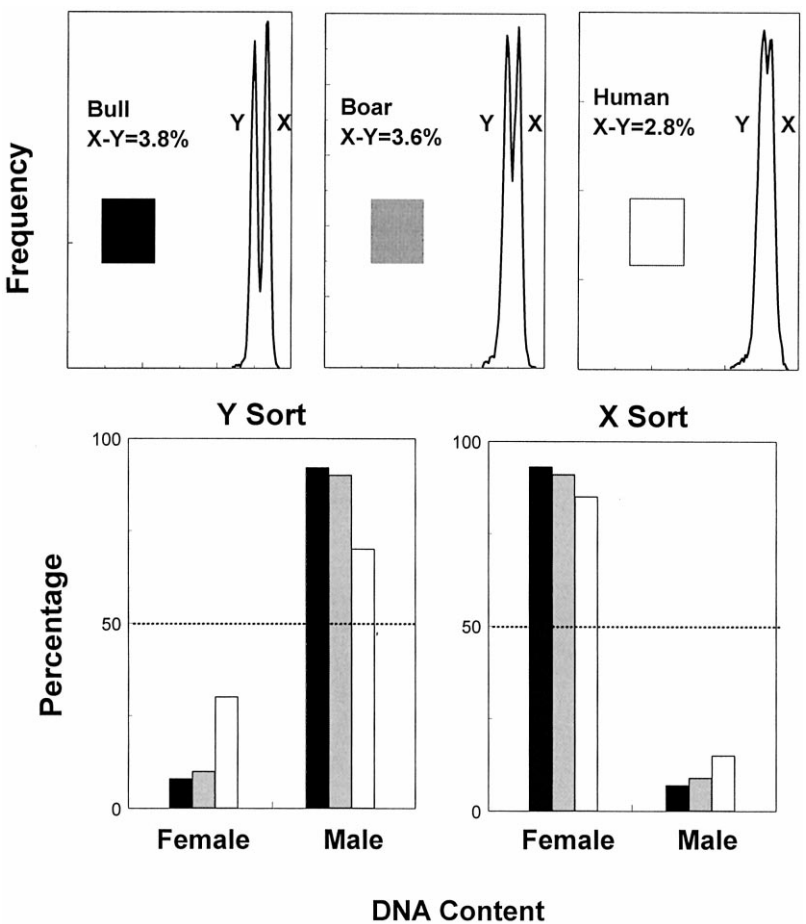

Fig. 3. The relative skewing of the sex ratio that can be obtained in four common species. The bar graph illustrates the approximate shift obtainable under standard sorting conditions of standard-speed or high-speed sperm sorting instrumentation.

application of sexed sperm in the cattle industry using high-speed sperm sorting technology. Based on these field results it is anticipated that sexed sperm will be available for commercial use by 2001. Fig. 3 illustrates the current technology for several species.

7. Freezing of sorted sperm for use in producing offspring in cattle and swine

The use of frozen semen as an option to fresh semen in conjunction with the sorting technology adds an important commercial dimension to the process, since frozen semen is highly important to widespread use of the technology, particularly in the cattle industry. Schenk et al. (1999) report the successful freezing of sperm after sorting with an average post-thaw motility of 30–35%. This is similar to results achieved by Johnson et al. (1996) in which an effect of sorting was shown with an average post-thaw motility of 35% and percentage of intact acrosomes of 40%. Seidel et al. (1999) have recently reported the production of calves from the use of sorted and frozen bull sperm. In several trials with sorted frozen sperm inseminated in the uterine horns, successful pregnancies were achieved using 1.5×10^6 frozen–thawed sperm. Pregnancies and/or calvings showed that the sex of offspring was essentially as predicted and approached 90% female or male depending on what sex of sperm was used.

Recently we have successfully frozen sorted boar sperm. The semen was thawed and used for surgical insemination. Viability assessments demonstrated that about 30% of the sperm could survive both sorting and freezing based on motility estimates. Very preliminary results with frozen sorted boar sperm has produced embryos and also piglets born (Johnson et al., 2000a). These results are promising in that the use of frozen semen in specialized circumstances for reproducing animals of the desired sex could be of special significance because of logistics.

8. Is there hope for an alternative sexing method based on immunological factors?

A method to sex sperm on a large scale, at least a scale that could be applied to semen production practice at an AI Center would certainly be advantageous. The method most often referred to in this context is that of isolating a protein from the surface of the X- or Y-bearing sperm that is chromosome specific and thus sex specific. The theory is that if one is able to isolate such a marker, then an antibody could be developed to attach to that X- or Y-bearing population of sperm. The assumption is that the use of affinity chromatography or magnetic bead separation would provide a batch separation process for separating X- from Y-bearing sperm and be readily adaptable to AI Center use. In order to determine if such a surface protein exists on boar sperm, a study was conducted in collaboration with colleagues in the Netherlands. Boar sperm was sorted for X or Y populations at purities of 90%. The sorted sperm were flown to the Netherlands where the proteins were isolated and characterized. Approximately 1000 proteins were mapped on the sperm surface. However, no difference could be detected between the proteins isolated from X sperm versus Y sperm. These results have led us to conclude that no sex-specific protein exists on the surface of the sperm (Hendriksen, 1999; Hendriksen et al., 1996).

Recently a new noninvasive immunological procedure for sexing sperm has been reported (Blecher et al., 1999). The method is based on the hypothesis that sex-specific proteins are more highly conserved than non-sex-specific proteins. Although the process shows some promise its efficacy has not been fully tested. Immunological procedures represent the best hope for seeking to prepare large amounts of sexed sperm in batch form thus providing an avenue for large-scale production and use.

9. Conclusions

The current state-of-the-art in sexing of mammalian sperm for offspring production is presented. The method is based on the differential amount of DNA (the only known difference between the X- and Y- chromosomes) being present in the X- and Y-chromosome-bearing sperm which is used as a marker to sort the X and Y populations. The use of a high-speed sorter modified for sorting sperm and adapted with an orienting nozzle is capable of producing up to 11 million sperm per/h of X sperm in our laboratory with 85% to 90% purity. More routine sorting can produce 6 million sperm/h where both X- and Y- sperm are needed. Additionally, even faster sorting rates (18 million/h) can be achieved albeit at a lower purity of X sperm (75%). It is anticipated that further refinements of current optical technology may increase the purity achieved at high sorting rates. Refinement of the high-speed sperm-sorting system is likely to lead to even greater production rates of X- and Y- sperm. High-speed sorting system pressures do not appear to affect fertilization significantly. The current technology for sorting sperm by flow cytometric sorting into X and Y populations at 90% purity of X or Y sperm is adaptable to virtually all mammalian species including human beings. Sexed sperm on demand should be on the market for livestock within the next 2 years. Currently, sperm sorting by this basic method is also being conducted in human clinical trials under the trademark MicroSort® (Fugger, 1999). At the present time the flow cytometric sorting method developed in 1989 (Johnson et al., 1989) and modified for high-speed sorting (Johnson et al., 1999) with an orienting nozzle provides the only fully validated means of preselecting mammalian offspring for sex.

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